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Phylogenetic characterization of subgingival plaque associated with aggressive or chronic periodontitis

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Abstract

Background and aims: Most of the so far identified bacterial species and phylotypes in the human oral cavity can be assigned to one of 11 phyla of the domain *Bacteria* and within these to 18 different classes. However, more than 50% of the oral bacteria cannot yet be cultivated and information about their relevance with respect to oral health is scarce. The aim of this study was to investigate the composition of subgingival plaque associated with advanced periodontitis by quantitative microscopic single-cell analysis using a panel of rRNA probes with specificity to large taxonomic units (classes, genera, families). The approach extends the more conventional use of probes that are directed against species that have been implicated in earlier studies by culture analyses in the etiology of periodontal diseases. One of the focal question asked was whether it will be possible to detect nearly all bacteria present in subgingival pockets rather than the 20-30% that can be covered by species-specific identification assays. A secondary aim was the monitoring of the changes in biofilm composition caused by antibiotic treatment directed against Gram-negative anaerobes.

Methods: Forty-five subgingival plaque samples from 16 patients suffering from advanced chronic or aggressive periodontitis were examined. Four patients underwent antibiotic treatment and were re-investigated 3-5 month after therapy. Fluorescent in situ hybridization (FISH) was used to investigate the prevalence and abundance of bacteria from 13 of the classes known to colonize subgingival pockets. To this end new 16S or 23S rDNA probes were designed, validated, and applied together with previously described probes in seeking as complete coverage as possible of the subgingival microbiota. This was accomplished either by using single probes, or by working with two or more complementary probes.

Results: The mean number of identified bacteria from 10 of the 13 classes varied between 10^5 and 10^6 per ml sample. Much lower numbers, around 10^4, were counted for *Capnocytophaga*, the only oral genus of the class *Flavobacteria,* whereas *γ-Proteobacteria* and TM7 were present in only few samples at concentrations barely above the detection limit. The prevalence of members from the 11 other classes varied between 68% and 100% and exceeded 90% in seven of these taxonomic units. Overall, the sum of the FISH-detected bacteria and the total number of detectable bacteria using a universal marker corresponded well, indicating that the employed probes covered the most abundant microbiota very well. Particularly noteworthy were the very high prevalence and density of bacteria

from the phylum of *Synergistetes,* especially from cluster A, which so far harbors only non-cultivable phylotypes. Among the *β-Proteobacteria* detected by probe β-42a a group of bacteria was frequently present, which could not be identified with our current set of phylogenetic probes. A similar result was obtained for bacteria belonging to the genus *Prevotella*, where much of the recognized diversity remained unexplainable by monitoring the most commonly studied *Prevotella* species. As expected the class of *Bacteroidia* with the major genera *Porphyromonas*, *Tannerella* and *Prevotella* accounted for a large number of the total subgingival biofilm bacteria. *Spirochaetes* detected by the two new probes Trep-G1-679 and Tlema738 with specificity for clusters I-III and IV, respectively, were well represented in more than 80% of the samples. Among the *Firmicutes* bacteria streptococci and eubacteria occurred with similarly high prevalence and abundance as the spirochetes, whereas the peculiar Gram-negative group of the *Negativicutes* harboring primarily the *Veillonella* and *Selenomonas* species rarely reached very high densities. The phylum of TM7 was undetectable in almost all probes and seems unlikely that these still cultivated bacteria play a role in the disease of periodontitis as suggested in the literature. Similarly, *γ-Proteobacteria,* apart from the *Aggergatibacter actinomycetemcomitans* and presumably a few other unidentified *Pasteurellaceae* species, were not detected.

Comparing the effects of deep scaling and root planing in combination with antibiotic intervention on the FISH-detectable microbiota, it was seen that before the start of the treatment *Bacteroidia, Spirochaetes* and *Synergistetes* were the predominant bacterial classes, whereas after the therapy *Fusobacteria* and *Proteobacteria* predominated. The total number of detected bacteria had decreased from 10^6-10^7 before to 10^4-10^5 3-5 months after the termination of the antibiotic intervention. These data provide evidence for a significant shift in plaque ecology as a result of the therapeutic intervention.

Conclusions: The data collected in this study show that the phyla-, class-, or family-specific probes are useful to analyze the composition of subgingival plaque associated with periodontitis. The number of microorganisms stained by the universal bacterial probe EUB338 and the number of bacteria labeled by all probes of non-overlapping specifity were nearly identical, which proves that it is possible to cover essentially the entire subgingival microbiota with probes to 11 major clusters of bacteria. A particular striking finding was the very high prevalence and density of large non-cultivable *Synergistetes* bacteria, especially of cluster A, raising important questions about a possible role of these bacteria in the periodontal inflammatory process.

Introduction

The human oral cavity is colonized by more than 500 cultivable bacterial species (Moore & Moore, 1994). These live attached to teeth and epithelial surfaces forming complex biofilms known as “plaque”. Young supragingival plaque contains mostly Gram-positive cocci and rods, whereas subgingival plaque from periodontal pockets is characterized by a high proportion of Gram-negative bacteria and, compared to supragingival plaque, a greatly increased concentration of motile rods. Some of these bacteria are thought to have an important role in the pathogenesis of periodontitis (Socransky & Haffajee, 1992; Socransky et al., 1998). However, more than 50% of the oral microbiota cannot yet be cultivated (Wilson et al., 1997).

The last 15 years have witnessed the emergence of a new field of research in microbiology ecology aiming at a better understanding of the diversity non-cultivable (or not yet cultivable) bacteria. To this end the16S ribosomal DNA (rDNA), 23S rDNA or the 16S–23S rDNA internal transcribed spacer region was targeted directly within the biomass using universal DNA primer pairs, PCR amplification of the primer-targeted DNA, and sequencing the cloned amplicons (Giovannoni et al., 1988; Amann et al., 1995). Applied to different forms of dental plaques such studies have led to identification of numerous new phylotypes of which, apart of their partial ribosomal RNA (rRNA) gene sequence little or nothing is known (Paster et al., 2002; Munson et al., 2004; Aas et al., 2005; Kumar et al., 2005; de Lillo et al., 2006; Colombo et al., 2009). These sequences have found their way into exponentially growing data banks, which nowadays form the backbone of the evolutionary tree of microorganisms. In this evolutionary tree, all bacterial species (cultivable) and phylotypes (only rDNA sequence available) from the human oral cavity were found to belong to one of 11 phyla and within these largest subdivisions of the domain *Bacteria,* to one of 18 classes.

Earlier studies from this laboratory on the composition and diversity of human subgingival and supragingival plaques could never identify more than approximately 30-50% of the microscopically detectable bacteria, regardless whether culture, immunofluorescence (IF), or fluorescence in situ hybridization (FISH) was used for identification and enumeration of the cells (Gmür & Guggenheim, 1994a; Gmür, 1995; Gmür et al., 2004). With their enormous diversity non-cultivable oral treponemes (Moter et al., 1998; Moter et al., 2006) are thought to account for some of these 50-70% of unknown bacteria, but it was evident from these earlier data that bacteria from other phyla must account for the majority of the unkowns. Therefore, we decided to combine in the present study a sensitive single cell

detection assay, FISH, with the application of a large panel of 16S and 23 rRNA oligonucleotide probes that comprises not only probes directed to single species or phylotypes as in most studies, but, in addition, probes with specificity for large taxonomic groups such as whole classes or families of bacteria.

As the principal source of phylogenetic information on the currently known oral microbiota, the Human Oral Microbiome Database (HOMD) was selected (Chen et al., 2010; Dewhirst et al., 2010). This database contains 16S rRNA gene sequence information and a taxonomic hierarchy listing of all oral species and phylotypes. These are assigned to 18 classes of the domain *Bacteria*, of which 13 were targeted in full or in part by our panel of oligonucleotide probes The HOMD database served us as a guideline in selecting the taxonomic units to which new probes were designed or existing probes were retrieved from the literature. All these probes were then used to investigate both the prevalence and abundance of the targeted bacteria in 45 samples from 16 patients with advanced chronic or aggressive periodontitis. The data indicate that it is indeed possible with this single-cell-staining approach to identify nearly all of the microscopically detectable bacteria at class or family level. Furthermore, pilot data are reported from a small group of four patients with 10 test sites who underwent antibiotic therapy following baseline examinations and were re-examined 3-5 month after the completion of the antimicrobial intervention.

Materials and Methods

Patients, sample collection, and sample processing prior analysis

Subgingival plaques samples (n = 43) were obtained as described previously (Gmür et al., 1989a) with three paper points per site from the deepest periodontal pockets of three quadrants of 16 patients (8 females). Two further sites of one patient concerned sites with peri-implantitis, both of which were sampled like the periodontitis affected sites. The clinical characteristics of the patient group are delineated in Table 3 and Table 4. All patients, including the one with peri-implantitis, suffered from advanced chronic or aggressive periodontitis and were undergoing periodontal treatment either at the Clinic for Preventive Dentistry, Periodontology and Cariology of the University of Zürich or at private practices. The patients had been referred to the Institute of Oral Biology for microbiological testing at the sampled sites. None of the patients had an antibiotic therapy within three month prior to sampling.

Following deep scaling and root planing four patients underwent antibiotic treatment with 3 x 2 tablets per day of Rodogyl for 10 days (composite tablet of 125 mg metronidazole and 750000 IU spiramycin, Sanofi-Aventis, France). From these patients 10 sites were re-analyzed microbiologically 3-5 months following the completion of the antibiotic intervention. Re-sampling was performed using the same procedures as at baseline.

Paper points from each individual site were collected in Eppendorf tubes in 600 µl 0.9% NaCl, containing protectRNA™ RNase inhibitor (RNAI; R-7397, Sigma-Aldrich, Buchs, Switzerland) at a dilution of 1:500 (Gmür & Lüthi-Schaller, 2007). The samples were processed immediately as follows: To disrupt plaque aggregates they were first sonicated at the maximum setting with an ultrasonic probe (Sonifier B-12, Branson Sonic Power Company, Danbury CT, USA) for three seconds on ice. The samples were then centrifuged (16'000 x *g*) for 10 min and the supernatants were aspirated carefully to a remaining volume of approximately 10 µl. If the pellets were stained red due to large amounts of erythrocytes, they were re-suspended for 5 min in 500 µl 0.83% NH_4Cl (Gmür & Guggenheim, 1983), re-centrifuged and washed once with 0.9% NaCl at room temperature. Otherwise the pellets were directly re-suspended in 500 µl of 4% PBS-buffered paraformaldehyde (PFA) and

fixed for 20 min at 4 °C. The sterile physiological phosphate buffer (PBS, pH 7.2) consisted of 7.5 g NaCl, 1 g Na_2HPO_4 and 0.4 g NaH_2PO_4 x H_2O per 100 ml of nanopure water. The suspension-fixed samples were washed once more in 0.9% NaCl and the supernatants aspirated to a residual volume of 50 µl. Finally 150 µl of nanopure water and 200 µl ethanol were added and the samples vortexed and stored at -20 °C until further use.

Cultivation of reference strains for the specificity testing of oligonucleotide probes

Reference strains from the OMZ strain collection were cultured on Columbia Blood Agar (Oxoid, Ltd., Basingstoke, Hamps., UK) and in fluid universal medium (Gmür & Guggenheim, 1983) + NAD (10 µg/ml) + 5% fetal bovine serum at 37 °C under anaerobic conditions or in case of *Pasteurella* strains in 10% CO_2 in air. Bacteria were harvested from the late log phase of growth and processed for FISH as described below.

Preparation of multiwell slides for FISH analysis

Plaques samples were defrosted, vortexted for 1 min and diluted in coating buffer (0.9% NaCl, 0.02% NaN_3, 0.00025% cetyltrimethylammonium bromide). The dilution factor varied between 1:2.5 and 1:8, depending on a microscopical estimate of the amount of bacteria present in the sample. Next, 10 µl of these suspensions were dropped into individual wells of 18- or 24-well microscope slides (epoxy coated multiwell slides with a well-diameter of 4 mm; Cel-line Associates, Newfield, NJ, USA). The slides were air-dried and fixed by a 20 min incubation at 4 °C in 4% PFA/PBS. Fixed slides were processed immediately for FISH or stored at 4-8 °C for a maximum of four weeks.

Preparation of slides for FISH: sample permeabilization and prevention of non-specific probe binding

If the FISH analysis was directed to Gram-negative bacteria there was no need to further permeabilize the cell wall of the bacteria. If it was directed to Gram-positive bacteria, or within the same well to both types of bacteria, further cell-wall permeabilization was sought as follows: the wells were exposed for 5 min to a 9 µl drop of lysozyme (Sigma L7651, 70000 U/mg, 1 mg/ml). In the case that lactobacilli had to be stained the wells were pretreated with lysozyme (1 mg/ml), achromopeptidase (Sigma, A 7550,

100000 U/mg, 1 mg/ml) and lipase (Sigma, L 1754, 1410 U/mg, 25 mg/ml) as described by Quevedo et al. (2011). Finally, to limit unspecific probe binding to the bacterial cell wall a drop of 9 µl Denhardt's solution (Fluka, Buchs, Switzerland), diluted 1:50 in PBS/RNAI, was distributed to all wells and the slides were incubated for 1 h at 37 °C (Gmür & Lüthi-Schaller, 2007).

Oligonucleotide probes and FISH

All probes used in this study are listed in the Supplementary Table S1. They were purchased from Microsynth (Balgach, Switzerland) as custom-synthesized oligonucleotide probes labeled either at the 5'-end with Cy3 or 6-FAM, or at both ends with 6-FAM. Oligonucleotide probes were designed according to Manz (1999) using the software *arb* (http://www.arb-home.de/; (Ludwig et al., 2004)). All probe sequences were in silico specificity-tested using rRNA sequence information from a) the Ribosomal Data Base Project II (http://rdp.cme.msu.edu/index.jsp; (Cole et al., 2009)), b) the SILVA 16S and 23S rRNA databases (http://www.arb-silva.de/; (Pruesse et al., 2007)), and c) the Greengenes website in combination with the HOMD data base (greengenes.lbl.gov; (Chen et al., 2010)). Previously described probes were retrieved from the literature as detailed in the Supplementary Table S1.

The lyophilized probes were dissolved in 100 µl TE buffer (1M Tris-HCl/100 mM EDTA, pH 8.0) and stored at -20 °C. From these primary solutions, 10x stock-solutions containing 50 ng probe per µl of TE buffer (Cy3-conjugates) or 150 ng/µl (FAM-conjugates) were prepared, aliquoted and stored at -20 °C. For FISH the probes were defrosted, diluted 1:10 in hybridization buffer composed of 0.9 M NaCl, 20 mM Tris/HCl pH 7.5, 0.01% sodium dodecyl sulfate (Fluka) and a probe-specific, optimum concentration of formamide. Optimum formamide concentrations had been determined in preliminary experiments for every probe (data not shown). The probe solutions were then added to the wells of the sample-coated multiwell slides (3-5 µl per well) and hybridization was performed for 4 h at 46 °C in 50 ml plastic centrifuge tubes as described by Züger et al. (2007). Following hybridization the slides were submerged for 30 min at 48 °C in washing buffer composed of 20 mM Tris/HCl pH 7.5, 5 mM EDTA, 0.01 % sodium dodecyl sulfate (SDS) and an amount of NaCl that depended on the formamide concentration of the hybridization buffer (Pernthaler et al., 2002). The washed slides were briefly dipped in ELISA washing solution (0.9% NaCl, 0.05% Tween 20, 0.02% NaN_3) and nanopure water before being air-dried in the dark. Finally, the slides were covered with 40 µl mounting fluid and a

coverslip. The mounting fluid consisted of 90% ultra pure glycerol (Invitrogen, Basel, Switzerland), 10% 10x PBS, and 25 mg/g Diazabicyclo[2,2,2]octane. Stained slides were stored at 4 °C in the dark.

Quantitative evaluation of samples stained by FISH

An Olympus BX60 microscope (Olympus Optical (Switzerland)) equipped with phase-contrast optics, an HBO 103 W/2 mercury photo optic lamp (Osram, Winterthur, Switzerland), two Olympus filter sets U-MNIBA and U-MA41007 for 6-FAM/FITC and Cy3 fluorescence, respectively, and 5100V2 (Chroma Technology Corporation, Bellows Falls, VT, USA) for combined FITC/TRITC fluorescence was used to analyze the samples. The wells of the multi-well slides were read at a 1000x magnification by the author and a technician. At least 10 viewing fields per well were assessed; if the density of positive bacteria was very low more wells were evaluated. From these countings the number of positive bacteria per ml of sample solution was calculated (Gmür & Guggenheim, 1994a).
Fluorescence intensity was graded using an arbitrary five-step scale, where "– " (no fluorescence above background) and 1+ (very faint fluorescence) were considered negative signals, and 2+ (weak), 3+ (strong) and 4+ (brilliant fluorescence) were considered positive signals. The detection limit of the FISH assay was approximately 5×10^2 bacteria per ml, if samples contained low numbers of bacteria and therefore were assessed at low sample dilutions. For high density samples the detection limit was 10x higher.

Microscopic images were taken with an Olympus E510 camera and stored on an iMac G5 personal computer (Apple, Cupertino, CA, USA). The original photos were processed only for a contrast improvement, cropping, and bar placement using iPhoto 6.0.6 (Apple) and Photoshop 7.0.1 (Adobe, San Jose, CA, USA)

Results

Characterization of the study group

Table 3 summarizes the main clinical characteristics of the patient group. Most patients (80%) suffered from advanced chronic periodontitis. Two individuals had local aggressive and one generalized aggressive periodontitis. The study group had a balanced sex distribution, whereas age distribution was heterogeneous (Table 3, 4). Probing depths at test sites varied between 4 and 12 mm with a means between 6 and 7 mm in both males and females. Presence of pus was three times more frequent in females than in males, but overall affected only 20% of the test sites (Table 4).

Detection of bacteria from the major phyla known to colonize the human oral cavity

Table 2 delineates the major classes of *Bacteria* and their subordinated families and genera that were investigated in this study. Both the prevalence and abundance of the detected bacteria belonging to these 13 classes are shown in Figure 1. The number of bacteria from 10 classes exceeded on average 10^5 bacteria per sample. For these classes the 25 and 75 % quantiles of the box plots extended over approximately an order of magnitude. Broader variation in cell density, as seen for example with *Actinobacteria*, went along with reduced prevalence. Very low prevalences were observed for *γ-Proteobacteria* and TM7*,* whereas *Flavobacteria* (in essence *Capnocytophaga* sp.) could be identified at about 2/3 of the test sites at low to moderate levels of density. The last two columns of Figure 1 show a comparison of the number of bacteria labeled by the universal probe EUB338 with the sum of the bacteria identified by all the probes used to cover the 13 aforementioned classes. Strikingly, the two box plots are almost identical, which indicates that the employed probes covered very well the most abundant microbiota present in this collection of 45 samples.

Analysis of β-, γ- *and* ε-Proteobacteria

The class of *β-Proteobacteria* was studied using the 23S rRNA probe β-42a, which covers most *β-Proteobacteria.* In addition, it identifies the family *Pasteurellaceae*, which by other criteria belongs to the *γ-Proteobacteria.* Supplementary Table S3 summarizes results of preliminary FISH experiments performed with strains of various *Aggregatibacter* and *Haemophilus* species that in part do not figure in the SILVA 23S rRNA sequence data bank. The data confirmed the anticipated positive reaction of β-42a (and the negative reaction of γ-42a) and at the same time demonstrated the high specificity of these probes that differ by only a single nucleotide. In the clinical samples *β-Proteobacteria* were detected in over 90% of the samples at levels between 10^3 and $>10^6$ bacteria (mean of approximately 10^6 cells; Figure 2A). We used probes for *Eikenella corrodens* (Ecor224), *Neisseria* sp. (NEI224) and *Aggregatibacter actinomycetemcomitans* (Aact639) to target specific taxa belonging to the organisms reactive with the β-42a probe. *E. corrodens* was detected more frequently than the aerobic *Neisseria* sp. but overall both taxa revealed low prevalence and density. *A. actinomycetemcomitans* was found at only four test sites of two chronic periodontitis patients (SN and CP) with levels between 3×10^5 and 2×10^6 bacteria per ml of sample. It follows that the presence of these taxa could not explain the identity of the majority of the β-42a-positive bacteria detected in the 45 samples investigated.
γ-Proteobacteria were investigated using probe γ-42a, which reacts with most *γ-Proteobacteria* except the *Pasteurellaceae*. In addition, several with specificity for *Moraxella lacunata* et rel., *Moraxella osloensis, Legionella* sp., or *Pseudomonas aeruginosa* were applied. With none of these probes any positive cells were detected. *Acinetobacter baumannii* et rel. was only detected in two tested sites (patient ME and SN) in very low numbers (6.7×10^2 and 1.5×10^3; Table 6).
L-EPSI549 is a broad class-specific probe for *ε-Proteobacteria*. The only known family of the *ε-Proteobacteria* known to occur in to oral cavity is *Campylobacter* sp., which was investigated by the probe CAMP655. The data shown in Figure 2A confirm the presence of *Campylobacter* sp. at high prevalence and mostly high density exceeding on average 5×10^5 bacteria per ml of sample solution. As expected the EPSI549 positive cells were small straight or slightly curved rods (Figure 3A).

Analysis of Firmicutes *and* Actinobacteria

There are no comprehensive probes available for the phyla of *Firmicutes* (Gram-positive bacteria with a low G+C content) and *Actinobacteria* (Gram-positive bacteria with a high G+C content). Therefore the numbers of bacteria belonging to these phyla were estimated by using a panel of different probes with specificity for families, genera, species, or phylotypes. The results are summarized in Table 5. The most prevalent taxa from the phylum *Firmicutes* were *Streptococcus* sp.(from the "*Bacilli*" class), *Eubacterium* sp., and *Parvimonas micra* (both from "Clostridia"). Streptococci and the *Eubacterium* species reached on average quite high levels around $3x10^6$ cells per sample. In contrast, *P. micra* remained at lower density in most samples. Streptococci were counted in lower numbers when identified by the mitis group probe MIT446 compared to the pan-streptococcal probe STR405, which suggested the possible presence of moderate levels of streptococci not belonging to the mitis or anginosus groups. Lactobacilli, *Gemella* and *Lactobacillus salivarius* bacteria (belonging to the "*Bacilli*") were found with rather low prevalence (18, 23 and 12%, respectively) at intermediate (*Gemella*) to low (lactobacilli and *L. salivarius*) concentrations. Staphylococci and enterococci could not be detected at all, inspite of using four different probes with specificity for various species (see Supplementary Table S1).

The prevalence and density of *Eubacterium yurii*, *Filifactor alocis* and the *Eubacterium* sp. identified by probe EUB818, are outlined in Table 5. The EUB818-positive *Eubacterium* sp. and phylotypes (*E. brachy, E. infirmum, E. minutum, E. nodatum, E. sulci* and several uncultivated phylotypes) were detected most frequently, usually in high numbers. *F. alocis* was stained by two probes. One (Falo219) is specific for *F. alocis,* the other one (Falo+Eyur490) recognizes *F. alocis* in combination with *E. yurii.* Our data demonstrate that *E. yurii*, was present at intermediate densities whereas *F. alocis* reached high concentrations at positive sites, however the prevalence of both species was lower than the one of the EUB818-postive eubacteria.

The class of *Actinobacteria* within the phylum carrying the same name includes the oral species *Actinomyces oris, Actinomyces naeslundii, Actinomyces israelii*, *Actinomyces gerensceriae, Actinomyces meyerii, Actinomyces odontolyticus*, as well as taxa from the families *Bifidobacteriaceae* and *Coriobacteriaceae*. We investigated these actinobacteria using probe ACT476 with specifity for the *A. naeslundii* group (*A. naeslundii, A. oris, Actinomyces radicidentis* and several uncultivated

phylotypes), a panel of species-specific probes for *A. israelii, A. gerensceriae, A. meyeri,* and *A. odontololyticus,* and two probes detecting various species of *Bifidobacteriaceae* and *Coriobacteriaceae* (see Supplementary Table 2). The obtained results are summarized in Table 5. ACT476 stained bacteria were observed in two thirds of the sample with positive samples harboring on average 6x 10^5 cells. In comparison the prevalence of *A. israelii* and *A. meyerii* was relatively low (18% for *A. israelii* and 32% for *A. meyerii*). When present, *A. meyerii* was detected with similar density as ACT476-positive bacteria, whereas the concentration of *A. israelii* was by a factor of 6 lower. *A. odontolyticus, Bifidobacteriaceae* and *Coriobacteriaceae* were detected in about 10% of the samples and when present reached on average approximately 10^5 cells per ml of sample. *A. gerensceriae* could not be detected.

Results on bacteria belonging to the order *Selenomonadales* (ex *Veillonellaceae*) with the Gram-negative oral *Firmicutes* families *"Selenonmonaceae"*, *Acidaminococcaceae*, and *Veillonellaceae* (Marchandin et al., 2010) are summarized in Table 6. Bacteria stained by the two broader *Selenomonas* probes SEL1150 and SEL1469 and by the species-specific *Selenomonas sputigena* probe Sspu439 were detected in 55 to 67% of the samples and colonized positive sites at levels between 1 and 5x 10^5. The majority of Sel1469-positive bacteria had the characteristic crescent-shaped morphology of cultivable *Selenomonas* cells, however a noteable minority of positive cells were much more stretched, slightly bent and had tapered ends (Figure 3B). The taxonomy of these cells remains to be identified. *Selenomonas noxia* was targeted with the species-specific probe Snox474 and found with low prevalence (11%). *Dialister* bacteria (L-Dia434-2 positive) were observed in every fifth sample and in these reached approximately the same density as the *Selenomonas* cells, whereas, *Megasphera* (MEG1147) and *Schwartzia* organisms could not be detected. Finally, veillonellae were found with a rather low prevalence of 38% but when present reached numbers similar to those observed for the mitis group streptococci.

Analysis of Fusobacteria

The phylum *Fusobacteria,* which comprises only the two not yet officially published families "*Fusobacteriaceae"* and "*Leptotrichiaceae"*, was monitored with probe FUS664 with specificity for the "*Fusobacteriaceae"* and the two probes Fnuc133c and Lbuc668. Fnuc133c identifies members of the

species *Fusobacterium nucleatum* and of closely related species and phylotypes whereas Lbuc668 recognizes *Leptotrichia buccalis* plus closely related phylotypes. All fusobacteria counted with the probe FUS664 (prevalence of 98%) were in high numbers, which is showed by Figure 2B. Stained fusobacteria had the characteristic fusiform morphology with considerable heterogeneity in cell length (Figure 3C). *Fusobacterium naviforme*, a species from within the rather heterogeneous *F. nucleatum* group (*F. nucleatum* et rel.) was detected with a prevalence of 80% and on average colonized the samples with $>10^5$ bacteria. *L. buccalis* observed in only half of the samples and consistently accounted for less than 10^5 of the detected bacteria. The morphology of the spindle-shaped rods marked by the probe for *L. buccalis* (Lbuc668) varied considerably with regard to length and thickness (not shown). Only the large fat rods resembled cultured *L. buccalis* cells, whereas other, much shorter and thinner cells resembled morphologically *Fusobacteria* or *Capnocytophaga* cells which confirms that the probe must detect further non-cultivable phylotypes.

Analysis of Spirochaetes

There are no comprehensive probes available for the phylum *Spirochaetes*, nor the genus *Treponema.* The latter harbors all the oral species and phylotypes. In the present study we investigated the broad diversity of oral treponemes partially using a newly devised Cluster 1 probe that covers all taxa previously assigned to groups 1, 2 and 3 in Choi et al.'s (1996) classification. Part of this cluster are for example the cultivable oral species *Treponema denticola, Treponema vincentii, Treponema medium,* and *Treponema parvum*. Further we used a new probe, Tlema738, with specificity for treponema group 4 organisms that include *Treponema lecithinolyticum, Treponema maltophilum* and multiple uncultivated phylotypes. The prevalence of samples positive for cluster 1 and group 4 treponemes was high (82 and 75%) and cell numbers exceeded 10^6 cells per ml of sample suspension at positive sites (Table 6).

Analysis of Bacteroidia

The class of *Bacteroidia*, formed among others by the clinically important oral genera *Bacteroides, Porphyromonas, Tannerella,* and *Prevotella*, was targeted by probe CFB935, which, according to the HOMD database, should hybridize to the oral species and phylotypes from all these groups. Bacteria

positive with CFB935 are shown in Figure 3D. Apparently, their morphology is quite heterogeneous and includes coccis, small rods, and fusiform rods. Occasionally rather long, filamentous rods were also observed. Overall probe CFB935 stained bacteria in every sample in very high density (cell numbers between 10^6 and 10^7 per ml sample) as shown in Figure 2C. *Prevotellaceae*, monitored by the probe PRV392, were often detected in high numbers with a prevalence of 93% (Figure 2C). We used several species-specific probes to identify the *Prevotella* species *Prevotella denticola*, *Prevotella intermedia, Prevotella nigriscens* and *Prevotella tannerae*, but as shown by Figure 2C, these species accounted for only a small part of the cells stained by PRV392. From the family *Porphyromonadaceae* only three species from two genera were investigated. *Porphyromonas endodontalis* (31%) and *Porphyromonas gingivalis* (51%) showed only intermediate prevalence levels. *Tannerella forsythia* was by far the most prevalent of the three species and on average was detected with more than 2x 10^6 cells per ml of sample suspension.

Analysis of Flavobacteria *and TM7*

The class of *Flavobacteria* includes among oral bacteria only the family of *Capnocytophaga*. *Capnocytophaga* sp. were detected in 68% of the samples where they reached modest densities, on average slightly more than 10^5 bacteria per sample (Table 6). The bacteria of the class TM7 could not be detected.

Analysis of Synergistetaceae

Synergistetes form a class of bacteria that with very few exceptions could not yet be cultivated. Almost all of oral phylotypes described so far fall into two clusters designated Group A and B. Group A was investigated on one hand by the probe SYN-A1409 and on the other hand by the two probes SYN-A1-632 and SYN-A2-207 which detect clearly distinct subclusters of cluster A. Group B was studied by probe SYN-B1149. Cluster A exhibited high prevalence and high cell densities. Group B was much more rarely detected and if present colonized the site in only low numbers (Figure 2D). Subgroups A1 and A2 were both detected in 90% of the samples where they reached remarkably high densities. Images of these group A *Synergistetes* bacteria are shown in Figures 3E and 3F. They are large and

relatively thick bent rods, which due to their size and large surface area seem to predominate more than the mere cell number could tell.

Comparison of the detected microbiota before and after antibiotic treatment

Four patients with a total of 10 test sites, of which two were located at implants, were followed-up three to five months after antibiotic therapy. Figure 4 shows a comparison of the distribution of Gram-negative bacterial clusters before and after therapy. In the graphs the sum of the detected bacteria was normalized to 100%. Before treatment three of the four patients showed very similar subgingival biofilm composition with a predominance of *Bacteroidia*, *Synergistetes* and *Spirochaetes*, whereas *Proteobacteria* and *Fusobacteria* were much less abundant. In contrast one subject (patient WM) had test sites in which *Proteobacteria* predominated.

After the therapy *Fusobacteria* and *Proteobacteria* clearly dominated the subgingival biofilm, whereas bacteria of the *Bacteriodia* class were greatly reduced. *Spirochaetes* and *Synergistetes* were absent or just above the detection limit. Only at one site of patient DN *Bacteroidia* cells were still predominant.

A big difference between before and after the antibiotic treatment was also detected for the total number of bacteria. Before the antibiotic intervention average numbers of bacteria per sample were around 10^6-10^7, after the therapy the numbers were between 10^4 and 10^5.

Discussion

The purpose of this study was to investigate subgingival plaque composition by a single cell detection assay (FISH) with rRNA-targeted oligonucleotide probes specific for large taxonomic groups such as classes, families or genera of bacteria. This contrasts sharply with the approach that most studies monitoring plaque composition take when they use a number of species-specific detection assays for taxa considered to be of relevance to periodontal diseases (e.g. Ximenez-Fyvie et al., 2000; Boström et al., 2001; Haffajee et al., 2008; Haffajee et al., 2009; Papapanou et al., 2009; Tadokoro et al., 2010; Eick et al., 2011). We have followed this experimental concept of working with species-specific detection tools in previous studies as well (Gmür et al., 1989; Gmür and Guggenheim, 1994; Gmür et al., 2004) and were always stunned by how small a proportion of the total number of microscopically recognizable bacteria we actually could identify in the studied plaque samples. Hence our questions were: 1) can the approach of this study, namely combining probes with specificity for large taxonomic groups with selected probes of much narrower specificity increase the proportion of identifiable bacteria in subgingival plaque from approximately 30% to close to 100%, and 2) in the affirmative case, to what major classes, genera and perhaps species/phylotypes do these missing 70% belong. The approach used in this study has been followed previously for the analysis of complex environmental microbial communities of little known composition (Giovannoni et al., 1990; Morris et al., 2002; Amann & Fuchs, 2008). Periodontitis associated plaque is of comparable complexity to these communities but in contrast contains a number of cultivable and thus more or less readily analyzable species that are considered to be putative pathogens (Socransky & Haffajee, 2008). These species have been in the focus of research during the last 20 years and even beyond oral microbiology figure among to the best-studied bacteria. In sharp contrast, the other subgingival microbiota, including all the non-cultivables, have been neglected. The here presented data show that on average the sum of bacteria labeled by all probes of non-overlapping specifity and the number of bacteria identified by the universal bacterial probe EUB338 were almost identical. This demonstrates that it is possible to associate at the single cell level nearly all periodontal plaque bacteria to one of 11 major taxonomic units. The distribution of the subgingival microbiota confirms previous studies, which investigated plaque diversity by rRNA gene sequencing (Paster et al., 2001; Hutter et al., 2003; Kumar

et al., 2006; Dewhirst et al., 2010). However, unlike the present investigation, these studies yielded only qualitative data (detection of rDNA fragment for species/phylotype) or provided an rRNA gene detection frequency hierarchy (*Veillonella* sp. clone X042 > (more prevalent than) *Campylobacter gracilis* >*Streptococcus mitis* etc.).

What were the most abundant phylogenetic units in our study that are missed by the commonly used panels of target species applied in clinical studies (Haffajee et al., 2004; Kamma et al., 2004; Machion et al., 2004; Fischer et al., 2008; Dahlén et al., 2010) and in periodontal diagnostics (Gmür and Guggenheim, 1994; Luterbacher et al., 2000; Cionca et al., 2010)? To implement our alternative experimental design, we developed new 16S and 23S rRNA probes to classes and genera of the phyla *Firmicutes, Actinobacteria, Spirochaetes,* TM7 and *Synergistetes* in order to complement probes of more narrower specificity and used previously described probes to identify and quantitate *β-, γ-,* and *ε-Proteobacteria, Bacteroidia, Fusobacteria*.

Viewed over the whole study it was found that the phyla-, classes, and family-specific probes are useful to analyze the subgingival plaque associated with periodontitis altogether. One of the remarkable findings throughout this study was a very high prevalence and large cell numbers per sample, on average exceeding 10^6 cells, of *Synergistetes* bacteria. *Synergistetes* bacteria are large curved rods. We have recognized such bacteria readily in plaques samples for many years but could never identify them. Due to their size they account for a significant volume of the subgingival biofilm and present a large surface area towards other biofilm members and to the surrounding host cells. Using the same probes as in this study, *Synergistes* organisms have recently been recognized to colonize the outermost region of the subgingival biofilm where they are directly opposed to cells of the inflammatory host response (Zijnge et al., 2010; Zijnge et al., 2012). *Synergistetes* organisms are barely cultivable. They are anaerobic and Gram-negative (Hugenholtz et al., 2009) but otherwise little is known about their characteristics. In the oral cavity various clusters of *Synergistetes* have been recognized (Horz et al., 2006; Vartoukian et al., 2009). Cluster A contains currently over 20 oral phylotypes of which none could be cultivated yet (Vartoukian et al., 2007). In cluster B there is with *Pyramidobacter piscolens* (Downes et al., 2009) one cultivable oral species besides multiple non-cultivable phylotypes. Cluster C harbors the fastidious species *Jonquetella anthropi* with isolates from the peritoneum, wounds, cysts, and abscesses (Warren et al., 2005; Jumas-Bilak et al., 2007) as well as the oral cavity (Munson et al., 2002). In this study, Cluster B cells were detected in only four samples from four different patients, whereas cluster A bacteria were omnipresent. *J. anthropi* was not

targeted, but more recent preliminary tests with a species-specific probe have not yet yielded positive data (not shown). Oral phylotypes in cluster A branch into two separate subgroups which we designated A1 and A2. Due to the frequent identification of cluster A bacteria we decided to design two new FISH probes for the A1 and A2 subgroups. Based on the latest release (August 2011) of the RDP data bank the sequence recognized by our A1 probe (SYN-A1-632-FAM) is present in 32 uncultured phylotypes of which 30 were of oral origin, whereas the one detected by the A2 probe (SYN-A2-207-Cy3) yields 21 hits with 20 of these phylotype-sequences derived from oral samples. Our results with these new A1 and A2 probes showed that both subgroups colonized subgingival periodontitis-associated plaques with almost identical, very high prevalence and most often reach high density (on average beyond 10^6 cells per ml of sample). Since cluster A *Synergistetes* could not be cultivated yet, virtually nothing is known about their physiology or a possibly beneficial or pathogenic role towards the host. It has been proposed by Zijnge et al. (2010) and Zijnge et al. (2012), that their dense pallisade-like arrangement along the outer length of the biofilm could suggest an important role in host-biofilm interactions.

The class of the *β-Proteobacteria* is another phylogenetic lineage that seemingly has been underestimated in many studies. Based on the literature we would have expected few *β-Proteobacteria,* namely *E. corrodens*, whereas the Gram-negative cocci of the genera *Kingella* and *Neisseria* were considered unlikely colonizers of a subgingival biofilm due to their aerobic metabolism. *Neisseria* sp. were indeed barely found, *Kingella* sp. were not investigated. *E. corrodens* was observed with much lower prevalence than β-42a positive cells*,* leaving the identity of a frequently present group of *β-Proteobacteria* open. In part these unidentified β-42a positive bacteria belong with high probability to the family *Pasteurellaceae,* which among others includes the orally important genera *Aggregatibacter* and *Haemophilus*. The *Pasteurellaceae* belong to the *γ-Proteobacteria,* but at the targeted region of the 23S rRNA have the sequence of *β-Proteobacteria.* We verified this for several *Pasteurellaceae* species currently not covered by the Silva 23S rRNA sequence data base (Supplementary Table 3). The β-42a probe detected further rods fatter than those of *Aggregatibacter, Haemophilus*, or *E. corrodens.* These bacteria could not be identified with our current set of phylogenetic probes. With respect to the *ε-Proteobacteria* our data suggest that in among the bacteria stained by the probe EPSI549 there might exist a group of cells that was negative with the *Campylobacter* probe CAMP655, of which we had expected coverage of all oral *ε-Proteobacteria*. To verify this result future double-labeling test with both these probes as well as a probe to *Heliobacter*

will have to be done to confirm the possible presence of EPSI549^{+}/CAMP655^{-} bacteria in such clinical samples.

The third large phylum harboring apparently considerable numbers of unidentified bacteria was *Bacteroidetes*. This group of Gram-negative rods has drawn a lot of attention in periodontal microbiology, not least because Socransky and coworkers have studied the composition of various forms of periodontal disease extensively with a set of some 40 different DNA probes each targeting a different oral bacterial species (Socransky et al., 1994; Socransky et al., 2004) and then described their findings in form of color-defined complexes. In their scheme the red and orange complexes harbored the species most implicated in the etiology of periodontitis, whereas e.g. the yellow complex was composed of microorganisms that were found most frequently at stable or healthy site (Socransky et al., 1998). Strikingly, several taxa of the red and orange clusters belong to the *Bacteroidetes* and there to the class of *Bacteroidia,* which harbors the genera *Porphyromonas* (with *P. gingivalis*) and *Tannerella* (with *T. forsythia*) and *Prevotella* (with *P. intermedia*). In this study only *T. forsythia* was regularly present in high numbers, whereas the prevalence of *P. gingivalis* was a mere 50%. This result matches with the structural findings of Zjinge et al. (2010), where *P. gingivalis* was detected only in the outermost layer of the subgingival biofilm, whereas in contrast *T. forsythia* was found dispersed throughout the biofilm. A notable observation was that *Prevotella* sp. (PRV932) were present with high prevalence and at very high density. However, the four investigated *Prevotella* species, *P. denticola, P. intermedia*, *P. nigrescens* and *P. tannerae* could not account for the high density of PRV392^{+} bacteria, which forcibly leads to the conclusion that there must be a significant additional diversity within the genus *Prevotella*.

Vis-à-vis of *P. gingivalis* the species *P. endodontalis* is a somewhat forgotten subgingival taxon that, however, has been associated with periodontitis, especially the chronic variant (Tran et al., 1997; Kumar et al., 2003). This study detected *P. endodontalis* with rather low prevalence (31%) and thus cannot confirm such an association. Similarly, the class of *Flavobacteria,* which in the subgingival space is represented by the sole genus *Capnocytophaga,* was detected only in rather small numbers, not confirming the previously reported marked association with periodontitis (Hutter et al., 2003; Ciantar et al., 2005). However, it must be emphasized that, due to the low sample number, these findings should be interpreted cautiously.

There are no comprehensive probes available for the phylum *Spirochaetes,* nor the genus *Treponema,* which harbors all oral species and phylotypes. The broad diversity of the oral treponemes

had been assessed previously by FISH with seven different group-specific probes and a variety of species-specific probes (Moter et al., 1998). In the present study we used a new Group 1 probe that covers all taxa detected by the probes to groups I, II and III of Moter et al. (1998). In addition we investigated the samples with a new spirochetal probe targeted to the cluster formed by *T. lecithinolyticum* and *T. maltophilum.*

The new probes for the *T. lecithinolyticum* and *T. maltophilum* (Tlema738) and for the Group 1 (TrepG1-679) were detected quite often (prevalence of 75-82%) in high number ranging between 2x 10^6 and 8x 10^6. Their high prevalence is consistent with their association with periodontitis (Colombo et al., 2009) and their affiliation to the red complex (Socransky et al., 1998).

As expected the phylum of *Firmicutes* was well represented in the studied samples owing especially to Gram-positive *Firmicutes* taxa that included *P. micra*, the group of EUB818$^+$ *Eubacteria* and several species of mitis streptococci which however are known primarily as pioneer colonizers of the subgingival root surface and otherwise prefer a supragingival habitat. EUB818$^+$ *Eubacteria, P. micra,* as well as several *Actinomyces* species, in particular *A. oris/A. naeslundii* have been noted previously to be associated with periodontitis lesions, but generally have not been linked to the etiology of the disease (Tanner et al., 1996; Hutter et al., 2003; Dalwai et al., 2006; Haffajee et al., 2006; Colombo et al., 2009; Dewhirst et al., 2010). The exceptional Gram-negative class of the *Negativicutes* (ex *Veillonellaceae*) with the motile group of *Selenomonas* bacteria and their close relatives, was detected in three out of four samples but rarely these bacteria reached as high densities as for example the fusobacteria or *Synergistetes* group A organisms. One might speculate that their motile life style may lead to lower abundance in comparison to the local proliferation of sessile bacteria. Among the bacteria positive with the *Selenomonas* probe SEL1469 was a notable minority of positive cells, which did not show the characteristic crescent-shaped morphology of *Selenomonas*. They were much more stretched, slightly bent and had tapered ends. The taxonomy of these cells remains to be identified.

The class of *Actinobacteria* was mainly represented by bacteria detected by probe ACT476, which covers in particular the two closely related species *A. naeslundii* and *A. oris.* Interestingly, *A. israelii, A. odontolyticus,* and *A. gerensceriae*, frequently observed in high numbers in plaque from the gingival crevice (Gmür et al., 2004) were only a minor component or absent in the samples of this study and far less frequent than e.g. *A. meyeri*. However, they were still more frequently observed and bacteria of the TM7 phylum. This poorly characterized group, harboring only uncultivated bacteria, has been brought to the attention of oral microbiologists by Brinig et al. (2003) who detected TM7 bacteria by

PCR and FISH in human subgingival plaque and speculated about a possible role in the etiology of periodontal diseases.

As mentioned in the introduction a secondary aim of this investigation was to monitor in a few patients the effects of adjunctive antibiotic therapy directed specifically at anaerobic bacteria. Not all patients received the same type of treatment and hence the already small test group was reduced to the size of a case description. Nevertheless it is interesting to record the antibiotic intervention resulted in an obvious decrease of the biofilm mass from 10^6-10^7 to 10^4-10^5 bacteria per ml sample. This corresponds to the clinical observations made by the responsible dentist and corroborates other studies reporting the reduction of periodontal pockets and an overall increase in periodontal health after antibiotic intervention (Haffajee et al., 2003; van Winkelhoff, 2003; Herrera et al., 2008; Cionca et al., 2009). A shift in the composition of the colonizing bacteria was detected leading from a predominance of *Bacteroidia, Spirochaetes* and *Synergistetes* to an apparently less aggressive biofilm consisting predominantly of *Fusobacteria* and *Proteobacteria.* Although observed with an entirely different methodology and after a different antimicrobial regimen our observations correspond well with previously reported findings (Sefton et al., 1996).

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Tables and Figures

Table 1. Major groups of oral bacteria: Phyla and subordinated classes of oral bacteria known to colonize in the human oral cavity

	Phyla										
	Proteobacteria	*Firmicutes*	*Actinobacteria*	*Fusobacteria*	*Spirochaetes*	*Bacteroidetes*	*Chlamydiae*	*SR1*	TM7	*Synergistetes*	*Chloroflexi*
Classes	*α-Proteobacteria*	***Bacilli***[a]	***Actinobacteria***	***Fusobacteria***	***Spirochaetes***	***Bacteroidia***	*Chlamidiae*	*SR1*	**TM7**	***Synergistetes***	*Chloroflexi*
	β-Proteobacteria	***Clostridia***				***Flavobacteria***					
	γ-Proteobacteria	***Negativicutes***									
	δ-Proteobacteria										
	ε-Proteobacteria										

[a] Bacteria belonging to classes printed in bold font were investigated in this study

Table 2. Major oral classes of *Bacteria* and the most prevalent subordinated families/genera from which oral bacteria were investigated[a]

Classes												
β-Proteobacteria	*γ-Proteobacteria*	*ε-Proteobacteria*	*Bacilli*	*Clostridia*	*Negativicutes*	*Actinobacteria*	*Fusobacteria*	*Spirochaetes*	*Bacteroidia*	*Flavobacteria*	TM7	*Synergistia*
Neisseriaceae	***Pseudomonaceae***	***Campylobacteraceae***	***"Staphylococcaceae"***	***Eubacteriaceae***	***Selemonadales incertae sedis***	***Actinomycetacae***	***Fusobacteriaceae***	***Spirochaetaceae***	***Bacteroidaceae***	***Flavobacteriaceae***	**TM7 incertae sedis**	**Synergistaceae**
Eikenella	Pseudomonas	*Campylobacter*	*Gemella*	*Eubacterium*	*Selenomonas*	*Actinomyces*	*Fusobacterium*	*Treponema*	*Bacteroides*	*Capnocytophaga*	TM7	*Pyramidobacter*
Kingella	***Moraxallaceae***	*Heliobacter*	*Staphylococcus*	***"Peptostreptococcaceae"***	*Centipedia*	***Bifidobacteriaceae***	*Leptotrichia*		***Porphyromonadaceae***			*unclassified*
Neisseria	*Moraxella*		***Lactobacillaceae***	*Filifactor*	*Mitsuokella*	*Bifidobacterium*			*Porphyromonas*			*Synergistaceae*
	Acinetobacter		*Lactobacillus*	***Incertae Sedis XI***	*Schwartzia*	***Coriobacteriaceae***			*Tannerella*			
	Pasteurellaceae[b]		***Streptococcaceae***	*Parvimonas*	***Veillonellaceae***	*Atopobium*			***Prevotellaceae***			
	Aggergatibacter		*Streptococcus*	***Incertae Sedis XIII***	*Dialister*	*Eggerthella*			*Prevotella*			
	Haemophilus		*Lactococcus*	*Mogibacterium*	*Megasphera*	*Olsenella*						
			"Enterococcaceae"		*Veillonella*							
			Enterococcus									
			"Carnobacteriaceae"									
			Granulicatella									
			"Aerococcaceae"									
			Abiotrophia									

[a] The classification sceme is based on the hierarchy view published by the Ribosomal Database Project (http://rdp.cme.msu.edu/; September 2011). A more detailed summary of the taxa and phylotypes detected by the employed probes is listed in the supplementary Table S2.

[b] *Pasteurellaceae* belong taxionomically to the **γ-***Proteobacteria,* but with the probes β-42a and γ-42a used in this study they are grouped with β-*Proteobacteria.*

[a] Further taxa and phylotypes detected by the employed probes are listed in Supplementary Supporting Table 2.

Table 3. Characterization of plaque donors

Diagnosis [a]	Patient	Age	Sex	Sites sampled	Probing depth at test sites
Moderate local aggressive periodontitis [a]	**LA**	**26**	**female**	**1: 17dl; 2: 44db; 3: 34dl**	**1: 6 mm; 2: 6 mm; 3: 6 mm**
Severe local aggressive periodontitis and peri-implantitis	**DN** [b]	**37**	**female**	**1: Impl. 11b; 2: 21p; 3: Impl. 43b**	**1: 4 mm; 2: 5 mm; 3: 6 mm**
Mixture between generalized chronic and aggressive periodontitis	**SS**	**58**	**female**	**1: 12m; 2: 21mp; 3: 46mb; 4: 26mp; 5: 47m**	**1: 7 mm; 2: 7 mm; 3: 7 mm; 4: 6 mm; 5: 6 mm**
Local chronic periodontitis	**WO**	**49**	**male**	**1: 16mp; 2: 15mp; 3: 36mb**	**1: 5 mm; 2: 6 mm; 3: 8 mm**
Local chronic periodontitis	**SN**	**54**	**female**	**1: 48d; 2: 45mb**	**1: 6 mm; 2: 5 mm**
Local chronic periodontitis	**PA**	**54**	**female**	**1: 16m**	**1: 6 mm**
Local chronic periodontitis	**CV**	**75**	**male**	**pool (18d; 17d; 36d)**	**(8 mm, 8 mm, 5 mm)**
Local chronic periodontitis	**EL**	**53**	**male**	**1: 18mb; 2: 11mp; 3: 21mp**	**1: 5 mm; 2: 5 mm; 3: 5 mm**
Local chronic periodontitis	**GM**	**47**	**male**	**1: 26db; 2: 24db; 3: 21db**	**1: 9 mm; 2: 7 mm; 3: 6 mm**
Severe local chronic periodontitis	**CP**	**45**	**female**	**1: 17mp; 2: 16mp**	**1: 6 mm; 2: 5 mm**
Severe local chronic periodontitis	**KA**	**54**	**male**	**1: 25d; 3: 46d**	**1: 7 mm; 3:9 mm**
Severe local chronic periodontitis	**ME**	**60**	**female**	**1: 47db; 2: 36mb; 3: 26db**	**1: 11 mm; 2: 10 mm; 3: 8 mm**
Severe generalized chronic periodontitis	**WM**	**58**	**male**	**1: 14m; 2: 26m; 3: 31m**	**1: 5 mm; 2: 6 mm; 3: 6 mm**
Severe generalized chronic periodontitis	**RU**	**64**	**male**	**2: 34m; 3: 35m**	**2: 6 mm; 3: 9 mm**
Generalized chronic periodontitis	**SK**	**54**	**male**	**1: 17b; 2: 27mp; 3:37b**	**1: 6 mm; 2: 6 mm; 3:12 mm**
Generalized chronic periodontitis	**DS**	**50**	**female**	**1: 37mb; 2: 24dp; 3: 21db**	**1: 9 mm; 2: 6 mm; 3:6 mm**

[a] Classification according to Armitage (1999)
[b] Patient was examined twice 10 days apart

Table 4. Clinical characteristics of study group

	female (*n*=8)	male (*n*=8)
Mean age (range)	47.1 yr (26-60 yr)	58.5 yr (47-75 yr)
Mean bleeding [a] (range)	medium to low (medium to low)	medium to low (extensive to very low)
Pus [b]	15.4%	5.0%
Mean sulcus probing depth	6.3 mm	6.8 mm

[a] Two different bleeding indices (BoP, PBI) were used by the different clinicians examining the patients. For a common denominator they were categorized here into very low, low, medium and extensive.

[b] 100% stands for the number of sampled sites.

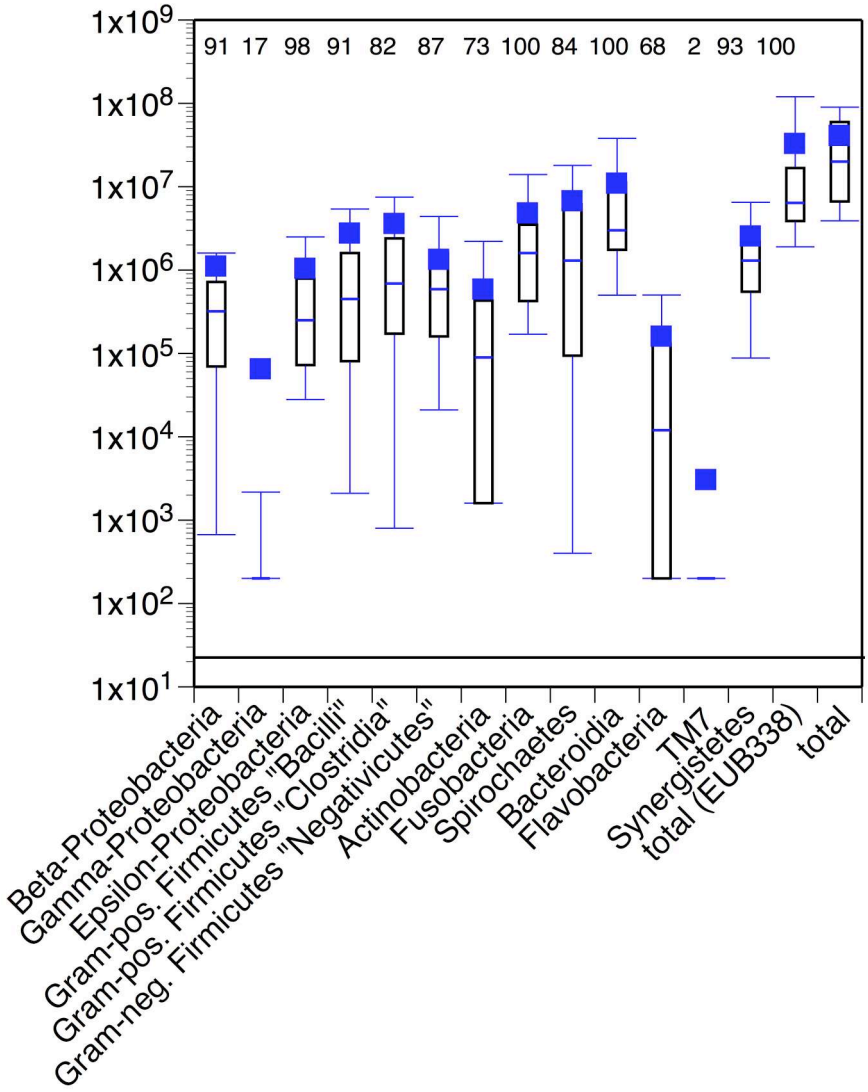

Figure 1. Boxplot describing the number of stained bacteria categorized by the different classes defined in Table 2.
"Total (EUB338)" shows the number of all bacteria stained with the comprehensive *Eubacteria* probe EUB338, whereas "total" represents the sum of all bacteria covered by the other columns of the figure. The horizontal line marks the detection limit of 200 bacteria per sample. The numbers in the upper part of the graph indicate the prevalence of the respective class with the total number of samples defined as 100%.

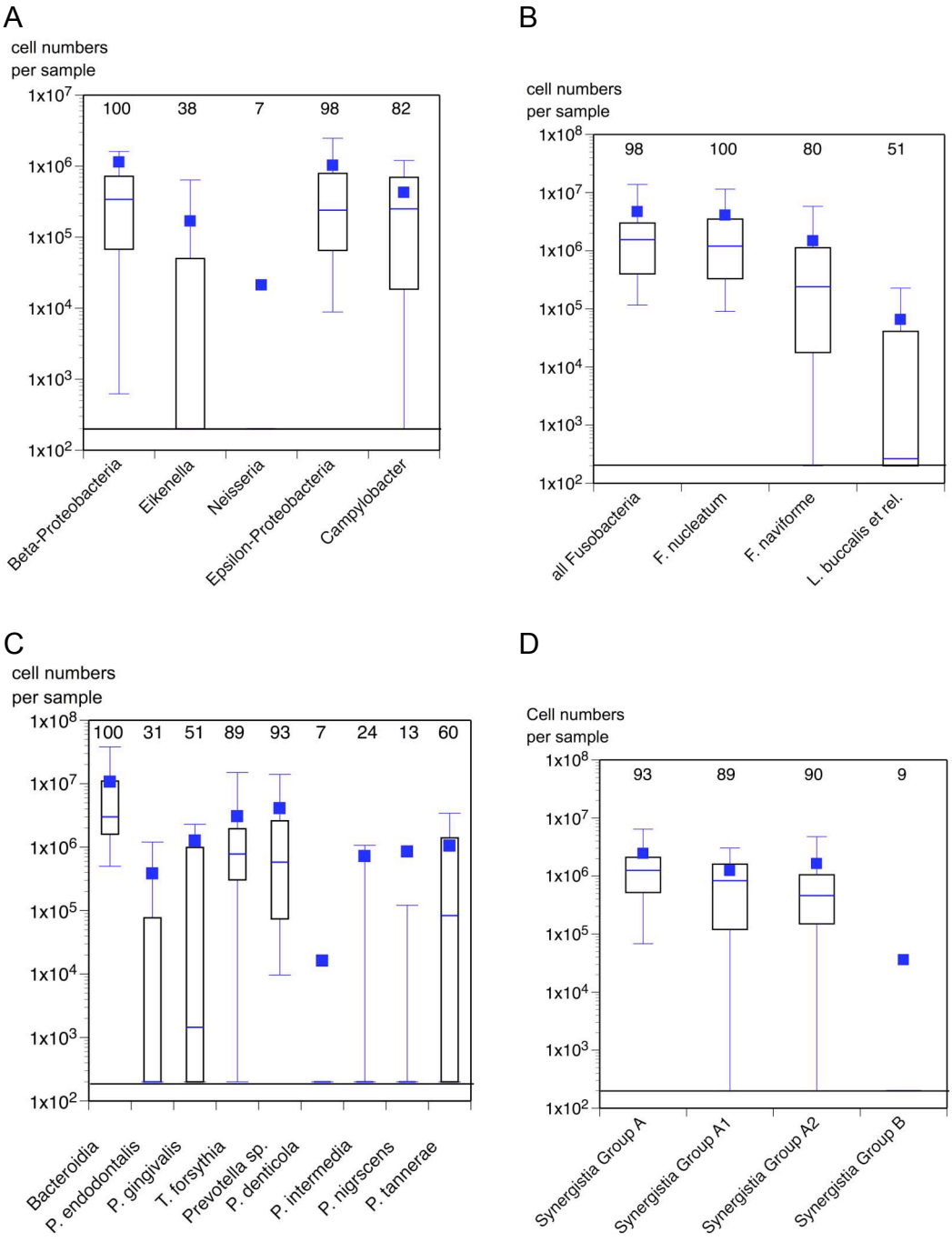

Figure 2. Quantitative distribution of the genera, species, or phylotypes from the Gram-negative classes of *Bacteroidia*, *Fusobacteria*, *β-* and *ε-Proteobacteria*, and *Synergistia* identified from subgingival plaque of advanced periodontitis.
The boxplots describe the distribution of FISH-labeled bacteria detected in the 45 test samples. Numbers above each box blot column indicate the prevalence of positive samples. **A.** Phylum *Proteobacteria* with two of its classes, *β-* and *ε-Proteobacteria. Eikenella* and *Neisseria* could only account for some 10% of the *β-Proteobacteria,* while the remaining 90% may in part belong to the order *Pasteurellacea.* The latter belong to *γ–Proteobacteria,* but β–42a and not γ-42a positive. *ε-Proteobacteria* are represented almost exclusively by the genus *Campylobacter.* **B.** Phylum of *Fusobacteria* made up of the genera *Fusobacteria* and *Leptotrichia*. *F. nucleatum/F. periodonticum*/*F. naviforme* account for all *Fusobacteria*. **C.** The first column shows the whole of *Bacteroidia* bacteria detectable with the broadly reactive probe CFB935, whereas the other columns identify the major *Bacteroidia* subgroup *Prevotella,* and several *Porphyromonas*, *Tannerella* and *Prevotella* species. **D.** Within the phylum of *Synergistetes* cluster A with the subgroups A1 and A2 predominated over cluster B bacteria that include the known oral species *Pyramidobacter piscolens*. The horizontal line marks the detection limit of 200 bacteria per sample.

Table 5. Prevalence and abundance at positive sites of Gram-positive bacteria

	Probe	**Prevalence**	**Mean (± SD)**
Firmicutes			
"Bacilli"			
Gemella sp.	GEM844	23%	3.4×10^5 ($\pm 3.7 \times 10^5$)
Staphylococcus sp.	L-STA478	0%	
S. aureus	Saur229	0%	
S. epidermidis	Sepi229	0%	
Lactobacillus sp.	LAB759	18%	8.5×10^4 ($\pm 9.7 \times 10^6$)
L. fermentum	Lfer466	4.5%	1.7×10^3
L. salivarius	Lsal1113	12%	5.5×10^4 ($\pm 3.7 \times 10^4$)
Streptococcus sp.	STR405	78%	2.9×10^6 ($\pm 9.7 \times 10^6$)
Mitis group + anginosus group streptococci	MIT446	53%	8.6×10^5 ($\pm 2.2 \times 10^6$)
S. constellatus +*S. intermedius*	Scoint172	0%	
Enterococcus faecalis	Efae193	0%	
"Clostridia"			
Eubacterium sp.	EUB818	80%	3.7×10^6 ($\pm 1.2 \times 10^7$)
F. alocis	Falo219	33%	2.8×10^6 ($\pm 4.5 \times 10^6$)
E. yurii and *F. alocis*	Falo+Eyur490	52%	3.3×10^5 ($\pm 4.0 \times 10^5$)
P. micra	Pmic740	44%	4.9×10^5 ($\pm 8.7 \times 10^5$)
Actinobacteria			
A. naeslundii et rel.	ACT476	66%	5.9×10^5 ($\pm 8.8 \times 10^{5)}$
A. israelii	Aisr999	18%	8.2×10^4 ($\pm 1.4 \times 10^5$)
A. gerensceriae	Ager481	0%	
A. meyerii	Amey1001	32%	5.3×10^5 ($\pm 9.8 \times 10^5$)
A. odontolyticus	Aodo993	11%	2.2×10^5 ($\pm 2.4 \times 10^5$)
Bifidobacteriaceae	BIF816	7%	1.2×10^5 ($\pm 2.0 \times 10^5$)
Coriobacteriaceae	COR284	9%	1.5×10^5 ($\pm 2.3 \times 10^5$)

Table 6. Prevalence and abundance at positive sites of bacteria from the Gram-negative classes: *Proteobacteria*, *Negativicutes*, *Spirochaetes* and *Flavobacteria*

	Probe	**Prevalence**	**Mean (± SD)**
Proteobacteria			
A. actinomycetemcomitans	Aact639	9%	3.0×10^6 ($\pm 5.2 \times 10^5$)
Moraxella sp	Mlac1462	0%	
A. baumannii et rel.	ACIN643	4%	2.2×10^3 ($\pm 5.9 \times 10^{2)}$
M. osloensis et rel.	Mosl636	0%	
L. pneumophila	LEGPNE-1	0%	
P. aeroginosus	PsearA1449	0%	
Negativicutes			
Dialister sp.	L-Dia434-2	20%	1.6×10^5 ($\pm 2.3 \times 10^5$)
Megasphera sp.	MEG1147	0%	
Selenomonas sp.	SEL1150	76%	5.6×10^5 ($\pm 7.3 \times 10^5$)
Selenomonas sp.	SEL1469	67%	4.4×10^5 ($\pm 7.5 \times 10^5$)
S. noxia	L-Snox474-2	11%	2.0×10^5 ($\pm 2.7 \times 10^5$)
S. sputigena	Sspu439	55%	4.7×10^5 ($\pm 8.9 \times 10^5$)
Schwartzia oral phylotypes	Schw1276	0%	
Veillonella sp.	VEI217	38%	7.1×10^5 ($\pm 1.0 \times 10^6$)
Spirochaetes			
Cluster 1 treponemes	TrepG1-679	82%	2.4×10^6 ($\pm 3.8 \times 10^6$)
T. lecithinolyticum + *T. maltophilum*	Tlema738	75%	7.3×10^6 ($\pm 1.7 \times 10^7$)
Flavobacteria			
Capnocytophaga sp.	CAP365	68%	2.4×10^5 ($\pm 4.7 \times 10^5$)

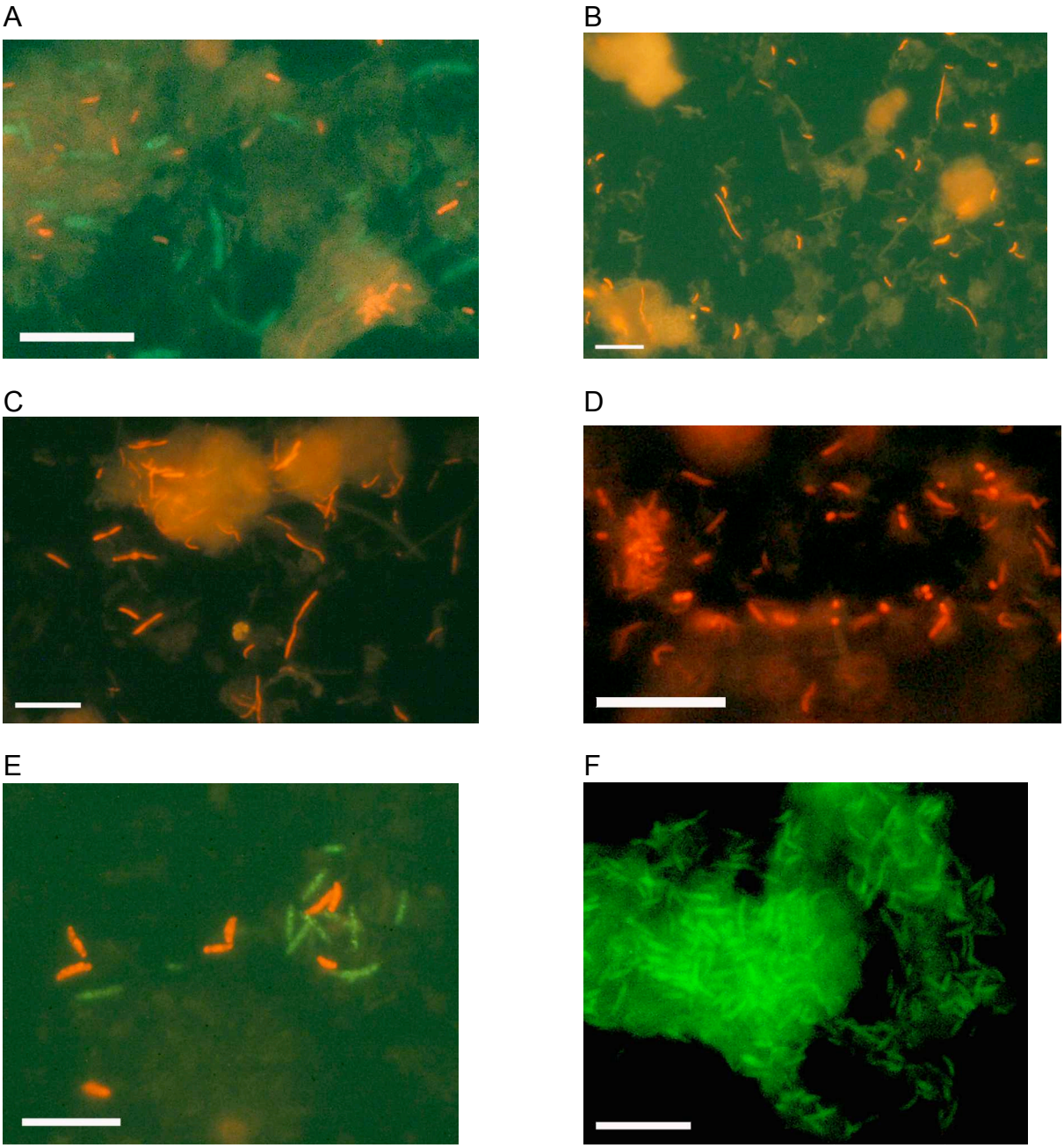

Figure 3. Representative FISH stainings of samples from various patients.
A Subgingival plaque stained with oligonucleotide probes L-EPSI549-1-Cy3 and SYN-A1-632-FAM. L-EPSI549-1-Cy3 labeled small straight or slightly curved rods that looked like *Campylobacter* sp., whereas SYN-A1-632-FAM identified much larger straight or curved rods belonging to the *Synergistia* Subgroup A1. The sample derived from site 1 of patient SK. **B.** Plaque stained with SEL1469-Cy3. In addition to the mostly small curved rods that are characteristic for *Selenomonas* sp., unidentified thin elongated curved rods with tapered ends were observed. Site 1 of patient LA **C.** Plaque intensively stained (3-4+) with FUS664-Cy3. The probe marked fusiform rods of variable length belonging to the *F. nucleatum/F. peridonticum/F. naviforme* cluster. Site 1 of patient LA. **D**. Sample hybridized with CFB935-Cy3. The probe stained heterogenic population of cells comprising coccis, straight short rods, bent rods, and thin fusiform rods. In most samples CFB935^{+} cells accounted for a comparatively high proportion of the total cell number. Site 1 of patient SK **E.** Subgingival plaque stained with the *Synergistia* probes SYN-A1-632-FAM and SYN-A2-207-Cy3. Both probes stained large bent rods with 4+ intensity. Positive cells displayed the characteristic morphology of Group A *Synergistia* organisms. Site 1 of patient SS. **F**. Plaque sample from site with peri-implantitis stained with SYN-A1409-FAM. The image shows a dense aggregate with a high proportion of positive rods exhibiting the characteristic morphology of Group A *Synergistia*. Site 1 of DN. Bars: 10 µm.

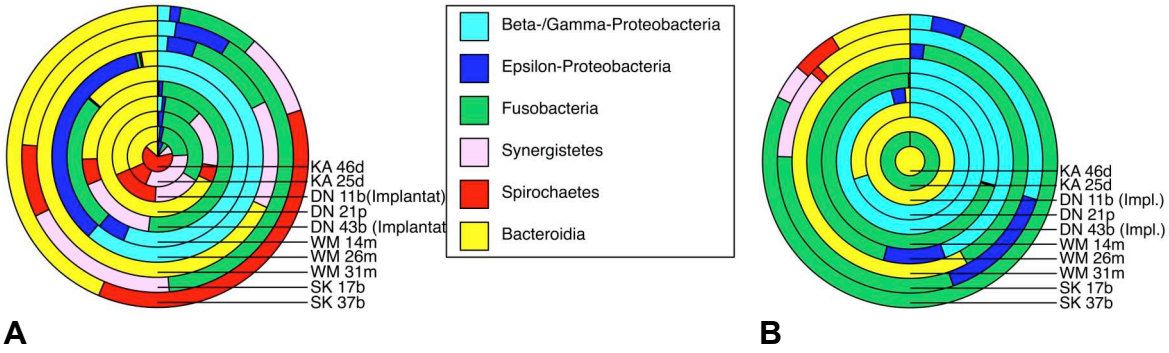

Figure 4. Proportion of bacteria from six major phyla or classes in periodontal pockets before and after deep-scaling and root-planing combined with antibiotic therapy (AT).
The sum of the detected bacteria was normalized to 100%. The data were collected from 10 sites of 4 patients. **A** Before AT *Bacteroidia* were very prominent at all but two sites of patients WM. *Fusobacteria*, *β-* and *γ- Proteobacteria*, *Synergistetes* and *Spirochaetes* were detected in high numbers as well, but not as uniformly. **B** After AT *Fusobactria* and *β-/γ-Proteobacteria* (β-42a+ cells) were predominant. The marked variability between patients and sometimes between different sites of the same patient is striking.

Supplementary table S1. Definition of the probes

Systematic position of target	Target	Probename	Target sequence (5' to 3')[a]	Label	Tm (°C)[b]	Pre-treat-ment[c]	% forma-mide	Reference
	Most *Eubacteria*	EUB338	GCTGCCTCCCGTAGGAGT	Cy3, FAM	55	No	25-50	(Amann et al., 1990)
Proteobacteria (Gram negatives)	*β-Proteobacteria*	Beta42a + Gamma42a-comp	GCCTTCCCACTTCGTTT GCCTTCCCACATCGTTT	Cy3 –	47	No	50	(Manz et al., 1995)
	Neisseria sp.	NEI224	CAGATATCGGCCGCTCGA	FAM	53	No	40	This study
	Eikenella corrodens	Ecor224	TCAGTTATCGGCCGCTCGA	Cy3	53	No	40	This study
	γ-Proteobacteria	Gamma42a + Beta42a-comp	GCCTTCCCACATCGTTT GCCTTCCCACTTCGTTT	Cy3 –	47	No	50	(Manz et al., 1995)
	Aggregatibacter actinomycetemcomitans	Aact639	CTCCAGACCCCCAGTATG	Cy3	53	No	40	This study
	Acinetobacter baumannii et rel.	ACIN643	CCCATACTCTAGCTVACCA	Cy3	50	No	40	This study
	Pseudomonas aeruginosa	PsaerA1449	GGTAACCGTCCCCCTTGC	FAM	55	No	40	(Hogardt et al., 2000)
	Moraxella lacunata et rel.	Mlac1462	TCGACCCCACCGTGGTGA	Cy3	55	No	40	This study
	Moraxella osloensis	Mosl636	CTAGCACCACCAGTATCAC	FAM	51	No	40	This study
	Legionellaceae Legionella sp.	LEGPNE1	ATCTGACCGTCCCAGGTT	FAM	50	No	used at 50[e]	(Grimm et al., 1998)
	ε-Proteobacteria	L-EPSI549-1	CAGTGATTCCGAGTAACG	Cy3	48	No	50	(Lin et al., 2006)
	Campylobacter sp.	CAMP655	CATCTGCCTCTCCCTYAC	Cy3	52	No	35	(Gmür&Lüthi-Schaller, 2007)
Fusobacteria	*Fusobacteria*	FUS664	CTTGTAGTTCCGCYTACCTC	Cy3, FAM	53	No	40	(Gmür et al., 2004)
	Fusobacterium nucletum	Fnuc133c	GTTGTCCCTANCTGTGAGGC	Cy3	55	No	40	(Guggenheim et al., 2009)
	Fusobacteria	Fnav1254	CTTCACAGCTTTGCGACTC	Cy3	51	No	25-35	(Zijnge et al., 2010)

	Leptotrichia sp.	Lbuc668	TACTCGTGCAGTTCCGTCC	Cy3	53	No	50	(Gmür et al., 2004)
Spirochaetes	*Treponema denticola* et rel.	TrepG1-679	GATTCCACCCCTACACTT	Cy3	48	No	50	(Zijnge et al., 2010)
	Treponema lecithinolyticum/maltophilum	Tlema738	GCGTCAATTATCTGCCGG	Cy3	50	No	20-35	(Guggenheim et al., 2009)
		L-Tlema738-2	GCGTCAATTATCTGCCGG	Cy3	50	No	40	(Guggenheim et al., 2009)
Bacteroidetes	*Bacteroides-Porphyromonas-Prevotella* subgroup of *Bacteroidia*	CFB935	CCACATGTTCCTCCGCTTGT	Cy3	54	No	40	(Daly&Shirazi-Beechey, 2003)
	Porphyromonas endodontalis	Pend740	CAGTGTCAGACGGAGCCT	Cy3	53	No	40	This study
	Porphyromonas gingivalis	L-Pgin1006-2	GTTTTCACCATCMGTCATC	Cy3, FAM	48	No	25	(Guggenheim et al., 2009)
	Prevotella sp.	PRV392	GCACGCTACTTGGCTGG	FAM	52	No	40	(Diaz et al., 2006)
	Prevotella denticola	Pden654	GCCGGCGTTGCGCGTACTC	FAM	60	No	25	(Gmür&Thurnheer, 2002)
	Prevotella intermedia	L-Pint649-2	CGTTGCGTGCACTCAAGTC	FAM	53	No	40	(Guggenheim et al., 2009)
	Prevotella nigrescens	Pnig657	TCCGCCTGCGCTGCGTGTA	Cy3, FAM	58	No	40	(Gmür&Thurnheer, 2002)
	Prevotella tannerae	Ptan630	GCCATCCCTGTGCGTACTC	Cy3	55	No	30-40	This study
	Tannerella forsythia	Tfor127	CTCTGTTGCGGGCAGGTTAC	Cy3	56	No	40	(Züger et al., 2007)
	Capnocytophaga sp.	CAP365	TCAGTCTTCCGACCATTG	Cy3	48	No	40	(Gmür et al., 2004)
Firmicutes (Low G+C Gram positives)	*Eubacterium brachy, E. infirmum, E. minutum, E, nodatum, E. sulci*	EUB818	CCGACACCTAGTGCTCAT	Cy3	50	No	25	This study
	Filifactor alocis, Eubacterium yurii	Falo+Eyur490	AGCCGGGGCTTTCTTKTA	Cy3, FAM	49	No	25-40	This study
	Filifactor alocis, Filifactor villosus	Falo219	GCGGGCTCATCTTTGTCC	FAM	52	No	40	This study
	Parvimonas micra et rel.	Pmic740	CTGAGCGTCAGTAAAAGTCC	Cy3	52	No	20-30	This study
	Most lactobacilli without *L. salivarius*	LAB759 + LAB759-comp	CTACCCATRCTTTCGAGCC CTACCCACGCTTTCGAGCM	Cy3, FAM	52	LAL	35	(Zijnge et al., 2010)

	Lactobacillus fermentum	Lfer466 + H448 (helper) + H484 (helper)	CCGTCAACGTATGAACAG TTACTCTCATACGTGTTC GCCGTGACTTTCTGGTTAAATA	Cy3	48	LAL	25	(Quevedo et al., 2011)
	Lactobacillus salivarius	L-Lsal1113-2	CTGGCAACTGACAACAAG	FAM	51	LAL	50	(Quevedo et al., 2011)
	Streptococci	STR405	TAGCCGTCCCTTTCTGGT	Cy3	50	Ly	40	(Thurnheer et al., 2001)
	Mitis group of streptococci, *S. anginosus*	L-MIT446-2	ACACYCGTTCTTCTCTTACAA	Cy3	48	Ly	40-50	(Guggenheim et al., 2009)
	Streptococcus constellatus, S. intermedius	L-Sco/int172-2	CAGTAAATGTTCTTATGCGGTA	Cy3, FAM	49	Ly	40	(Guggenheim et al., 2009)
	Enterococcus faecalis	Efae193	CGAAAGCGCCTTTCACTC	FAM	50	Ly	25	(Guggenheim et al., 2011)
	Staphylococcus sp.	L-STA487-2	CCGTGGCTTTCTGATTAGG	FAM	51	Ly	30-50	This study
	Staphylococcus aureus	Saur229	CTAATGCAGCGCGGATCC	Cy3	53	Ly	30-50	This study
	Staphylococcus epidermidis	Sepi229	CTAATGCGGCGCGGATCC	FAM	55	Ly	Used at 50[e]	This study
	Gemella sp.	GEM844	GCTGCAGCACTGATCTCT	Cy3	50	Ly	25	This study
Firmicutes (Low G+C Gram negatives)	*Dialister* sp.	L-Dia434-2	TTCGTCCCGRATCACAGA	FAM	49	No	40	(Gmür et al., 2004)
	Megasphera sp.	MEG1147	TGCGGCAGTCTCTCCTGA	FAM	53	No	50	(Suter et al., 2011)
	Selenomonas sp., *Centipedia periodontii, Schwartzia succinivorans*	Sel1150	CTCCGCGGCAGTCTCCTT	FAM	55	No	40	(Zijnge et al., 2010)
	Selenomonas dianae, S. flueggei, S. infelix, S. noxia, Centipedia periodontii	Sel1469	CCAGTCACCTTCCCCACC	Cy3	53	No	50	(Suter et al., 2011)
	Selenomonas sputigena	Sspu439	CGGTTTTCGTCCCGTGCA	Cy3	53	No	50	(Suter et al., 2011)
	Selenomonas noxia et rel.	L-Snox474-2	GATGGGTACCGTCATTRCCT	FAM	53	No	50-60	This study
	Schwartzia, oral phylotypes	Schw1276	GATGTTTTCTGGGGTTCG	FAM	48	No	Used at 25[e]	This study
	Veillonella atypica, V. dispar, V. parvula,	VEI217	AATCCCCTCCTTCAGTGA	Cy3, FAM	48	No	35, 40	(Thurnheer et al., 2004)
Actinobacteria	*Actinomyces naeslundii* et rel.	L-ACT476-2	ATCCAGCTACCGTCAACC	Cy3	51	Ly	40	(Gmür&Lüthi-Schaller, 2007)

Actinobacteria (High G+C Gram-positives)	*Actinomyces naeslundii* et rel.	L-ACT476-2	ATCCAGCTACCGTCAACC	Cy3	51	Ly	40	(Gmür&Lüthi-Schaller, 2007)
	Actinomyces naeslundii et rel.	ACT476	ATCCAGCTACCGTCAACC	Cy3, FAM	50	Ly	25-35	(Gmür&Lüthi-Schaller, 2007)
	Actinomyces gerencseriae	Ager481	CTTCAATTAACCCAGCTAC	Cy3	47	Ly	25	This study
	Actinomyces israelii	Aisr999	GTCTCCAGGAGCAGCCGG	Cy3	57	Ly	40	This study
	Actinomyces meyerii	Amey1001	CACGTCTCCGCACGCCCAG	Cy3	60	Ly	40	This study
	Actinomyces odontolyticus	Aodo993	GCASTGCCGCCGTGCATG	Cy3	57	Ly	50	This study
	Bifidobacterium sp., *Gardnerella vaginalis*	BIF816	CACATCCAGCRTCCACCG	Cy3	54	Ly	50	This study
	Coriobacteriaceae with *Atopobium* sp., *Collinsella* sp., *Eggerthella* sp., *Olsenella* sp.	COR284	GTCTCTCAACCCRGCTACCC	FAM	57	Ly	40	This study
TM7	TM7 oral phylotypes	TM7_571	CCRCCTACGCAACTCTTTAC	Cy3	52	No	40	This study
Synergistetes	unclassified *Synergistiaceae* group A	SYN_A1409	ACACCCGGCTCGGGTGGT	FAM	57	No	50	(Zijnge et al., 2010)
	unclassified *Synergistiaceae* subgroup A1	SYN_A1_632	GCACCTCAGTCTCAACTGC	FAM	53	No	30-40	This study
	unclassified *Synergistiaceae* subgroup A2	SYN-A2_207	CCTCCTCCAGCGCATCTC	Cy3	55	No	30-40	This study
	Pyramidobacter piscolens et rel.	SYN_B1149	TCGATGGCAGTCTCGCCG	Cy3	55	No	50	This study

[a] Bold labeled bases indicate the position of locked-nucleic-acids.

[b] Calculated by the formula Tm = 64.9°C + 41°C x ((number of G's and C's in the probe - 16.4)/length)

[c] Pretreatments: No: none; Ly: 5 min lysozyme; LAL: 5 min lysozyme and achromopeptidase followed by 30 min lipase

[d] Formamide concentration used in hybridization buffer.

[e] Used at the indicated formamide concentration. The optimum concentration was not determined due to the lack of appropriated strains or positive biofilm samples

Supplementary table S2. Definition of the probes using the HOT numbers

Systematic position of target	Target	Probename	HOT number of detected taxa (no mismatches)
	Most *Eubacteria*	EUB338	-
Proteo-bacteria (Gram-negatives)	*β-Proteobacteria* + *Pasteurellaceae* (γ-P.) + *Cardiobacteriaceae* (γ-P.)	Beta42a + Gamma42a-comp (23S)	014; 023; 092; 343; 476; 531; 545; 571; 577; 598; 610; 621; 633; 641; 646; 649; 669; 682; 706; 717; 718; 720; 737; 762; 764; 821; 828
	Neisseria sp.	NEI224	598; 610; 669; 737; 476
	Eikenella corrodens	Ecor224	577
	γ-Proteobacteria	Gamma42a + Beta42a-comp (23S)	477; 536; 554; 574; 612; 711; 731; 827; 833
	Aggregatibacter actinomycetemcomitans	Aact639	531
	Acinetobacter baumannii et rel.	ACIN643	554; 408
	Pseudomonas aeruginosa	PsaerA1449	536
	Moraxella lacunata et rel.	Mlac1462	833
	Moraxella osloensis	Mosl636	711
	Legionellaceae Legionella sp.	LEGPNE1	-
	ε-Proteobacteria	L-EPSI549-1	842; 580; 623; 748; 763; 044; 776; 812
	Campylobacter sp.	CAMP655	575; 623; 748; 763; 044; 776
Fuso-bacteria	*Fusobacteria*	FUS664	200; 690; 420; 698; 202; 201; 203; 370
	Fusobacterium nucletum	Fnuc133c	210; 220; 200; 420; 202; 203; 370
	Fusobacteria	Fnav1254	200
	Leptotrichia sp.	Lbuc668	563; 224; 214; 212; 213; 215; 223; 225; 392; 417; 462; 463; 498; 222;
Spiro-chaetes	*Treponema denticola* et rel.	TrepG1-679	584; 667; 805; 743; 226; 227; 228; 230; 231; 232; 234; 235; 236; 237; 238; 239; 242; 246; 247; 250; 251; 252; 253; 254; 255; 256; 508; 517; 518; 029
	Treponema lecithinolyticum/maltophilum	Tlema738	653; 258; 260; 490; 664
		L-Tlema738-2	653; 258; 260; 490; 664

Bacteroi-detes	*Bacteroides-Porphyromonas-Prevotella* subgroup of *Bacteroidia*	CFB935	280; 281; 365; 436; 503; 505; 507; 511; 516; 630; 787; 465; 272; 274; 547; 283; 273; 619; 275; 277; 278; 279; 284; 285; 395; 613; 286; 808; 553; 556; 560; 562; 583, 291; 298; 643; 658; 289; 665; 469; 378; 685; 794; 693; 705; 311; 288; 714; 303; 307; 795; 292; 296; 299; 300; 301; 302; 304; 305; 306; 308; 309; 313; 314; 315; 317; 376; 443; 472; 473; 474; 475; 515; 526; 781; 782; 820; 466; 572
	Porphyromonas endodontalis	Pend740	273; 285; 395
	Porphyromonas gingivalis	L-Pgin1006-2	619
	Prevotella sp.	PRV392	553; 556; 560; 562; 583; 291; 600; 298; 643; 658; 289; 665; 469; 378; 685; 794; 693; 705; 311; 288; 714; 303; 307; 795; 292; 296; 299; 300; 301; 302; 304; 305; 306; 308; 309; 313; 314; 315; 317; 376; 396; 443; 472; 473; 474; 475; 515; 781; 782; 820; 466; 572
	Prevotella denticola	Pden654	291; 685
	Prevotella intermedia	L-Pint649-2	643
	Prevotella nigrescens	Pnig657	693
	Prevotella tannerae	Ptan630	466
	Tannerella forsythia	Tfor127	613
	Capnocytophaga sp.	CAP365	337; 325; 627; 700; 323; 324; 326; 329; 332; 334; 335; 336; 338; 380; 412; 775
Firmicutes (Low G+C Gram positives)	*Eubacterium brachy, E. infirmum, E. minutum, E, nodatum, E. sulci*	EUB818	105; 467; 557; 673; 694; 091; 495; 103; 369
	Filifactor alocis, Eubacterium yurii	Falo+Eyur490	377; 539; 106
	Filifactor alocis, Filifactor villosus	Falo219	539
	Parvimonas micra et rel.	Pmic740	111; 110; 393
	Most lactobacilli without *L. salivarius*	LAB759 + LAB759-comp	529; 558; 568; 816; 817; 615; 838; 839; 819; 709; 716; 818; 749; 418; 424; 461; 051

	Lactobacillus fermentum	Lfer466 + H448 (helper) + H484 (helper)	608
	Lactobacillus salivarius	L-Lsal1113-2	756
	Streptococci	STR405	537; 543; 073; 576; 578; 594; 622; 638; 644; 398; 677; 686; 707; 721; 411; 728; 734; 745; 755; 758; 767; 768; 055; 056; 057; 058; 061; 064; 065; 066; 067; 068; 069; 070; 071; 074; 423; 431; 486
	Mitis group of streptococci, *S. anginosus*	L-MIT446-2	073; 622; 638; 398; 677; 707; 721; 411; 728; 734; 758; 767; 055; 056; 057; 058; 061; 064; 065; 066; 067; 068; 070; 071; 074; 423; 431; 486
	Streptococcus constellatus, S. intermedius	L-Sco/int172-2	576; 644
	Enterococcus faecalis	Efae193	604
	Staphylococcus sp.	L-STA487-2	567
	Staphylococcus aureus	Saur229	No match within HOMD !!
	Staphylococcus epidermidis	Sepi229	567
	Gemella sp.	GEM844	555; 626; 046; 757
Firmicutes (Low G+C Gram negatives)	*Dialister* sp.	L-Dia434-2	118; 843; 736; 119; 502
	Megasphera sp.	MEG1147	121; 122; 841
	Selenomonas sp., *Centipedia periodontii, Schwartzia succinivorans*	Sel1150	726; 124; 139; 125; 639; 130: 126; 133; 134; 136; 137; 138; 143; 146; 149; 388; 478; 479; 481; 501; 151
	Selenomonas dianae, S. flueggei, S. infelix, S. noxia, Centipedia periodontii	Sel1469	No match within HOMD !!
	Selenomonas sputigena	Sspu439	134; 143; 151
	Selenomonas sp. oral taxon	L-Snox474-2	130
	Schwartzia oral phylotypes	Schw1276	132; 135; 145; 148; 150; 155; 483
	Veillonella atypica, V. dispar, V. parvula,	VEI217	524; 160; 161; 158
Actino-bacteria (High G+C Gram-positives)	*Actinomyces naeslundii et rel.*	L-ACT476-2	688; 176; 746; 169; 170; 171; 175; 177; 179

	Actinomyces naeslundii et rel.	ACT476	688; 176; 746; 169; 170; 171; 175; 177; 179
	Actinomyces gerencseriae	Ager481	617; 178
	Actinomyces israelii	Aisr999	618
	Actinomyces meyerii	Amey1001	645
	Actinomyces odontolyticus	Aodo993	671
	Bifidobacterium sp., Gardnerella vaginalis	BIF816	701; 172; 180; 181
	Coriobacteriaceae with Atopobium sp., Collinsella sp., Eggerthella sp., Olsenella sp.	COR284	198; 407; 829; 586; 642; 195
	TM7 oral phylotypes	TM7-571	674; 723; 750; 199; 416; 810; 814; 579; 654; 806; 807; 809; 038
Syner-gistetes	*unclassified Synergistiaceae group A*	SYN-A1409	346; 349; 352; 353; 488; 350; 351; 355; 356; 437
	unclassified Synergistiaceae subgroup A1	SYN-A1-632	358; 359; 360; 361; 362; 363; 452; 453
			358; 359; 361; 362; 452; 453
			363
	unclassified Synergistiaceae subgroup A2	SYN-A2-207	
	Pyramidobacter piscolens et rel. Group B	SYN-B1149	

Supplementary table S3. Specificity of oligonucleotide probes β-42a and γ-42a					
Probe	*Species/Strain*				
	A. actinomycetem-comitans	*A. aphrophilus*	*A. segnis.*	*H. parainfluenzae*	*A. aphrophilus*
	JP2 (OMZ 295)	HK315 (OMZ 355)	HK84 (OMZ 356)	HK47 (OMZ 358)	HK322 (OMZ 359)
Aact639-Cy3	4+	–	–	–	–
γ-42a-FAM	–	–	–	–	–
γ-42a-Cy3	–	–	–	–	–
β-42a-Cy3	3-4+	3+	4+	4+	4+